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The Photosynthetic Apparatus of *Ectothiorhodospira halochloris*
2. Accessibility of the Membrane Polypeptides to Partial Proteolysis and Antenna Polypeptide Assignments to Specific Chromophores

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Bacterial Photosynthesis, *Ectothiorhodospira*, Membrane Topology, Antenna Polypeptides, Fluorescence, Circular Dichroism, Energy Transfer

E. halochloris thylakoids and spheroplasts were treated with trypsin, thermolysin or proteinase K to determine which proteins are exposed at the different membrane surfaces. Based on SDS polyacrylamide analysis, all 9 polypeptides are exposed on the cytoplasmic side. Only one (28 kDa) is accessible from the periplasmic side. This polypeptide is generally isolated as the H-subunit of the reaction centers of photosynthetic bacteria, but is in the case of *E. halochloris* rather isolated with the antenna (B 800/1020) (Steiner and Scheer, Biochim. Biophys. Acta **807**, 278, 1983).

Proteolysis is accompanied by a shift of the absorption band at longest wavelengths from 1020 to 960 nm (B 800/960), which upon standing is shifted further to 680 nm ("B" 800/680). The spectral changes are similar to the ones reported earlier for treatment with acid, and are also inducible with urea. The correlation of SDS-PAGE and absorption spectroscopy shows, that the chromophores absorbing at 1020 nm are transformed simultaneously with the degradation of the 6.5 kDa (= α) polypeptide.

Introduction

Much progress has been made in recent years in understanding the topology of photosynthetic membranes in purple bacteria. The techniques used included biochemical methods of different specifities and advantages like labelling with antibodies [1], radioiodonation [2], photoaffinity labelling [3], cross-linking experiments [4–6] or proteolytic digestion [7–9]. They also included diffraction methods, like high-resolution electron microscopy [10–12] and most recently x-ray diffraction of isolated reaction centers [13].

Most of this work was focused on only a small number of closely related bacterial species. Particular emphasis has been placed on the Rhodospirillales [14] *e.g. Rs. rubrum, Rp. spheroides, Rp. capsulata* and *Rp. viridis*. Much less is known about species from other genera [15], and their structural relations to the Rhodospirillales. We have recently begun to study the photosynthetic apparatus of *E. halochloris* [16], an alcalophilic and extremely halophilic bacteriochlorophyll *b* containing organism.

The main difference as compared to *e.g. Rp. viridis* is a second near-infrared absorption band besides the common one at 1020 nm, peaking at 800 nm with a shoulder at 830 nm. In a previous study [17], it was shown that the native ("high-pH") form (λ_{max} = 1020 and 800 nm) is reversibly transformed below pH 6.5 to a form absorbing at 960 and 800 nm ("low-pH" form). The 800/830 nm band remained unchanged by this treatment [17]. The pigments relating to the 960 nm band in the "low-pH" form are not very stable, and are oxidized irreversibly upon addition of more acid or even incubating at ambient temperature. The newly formed absorption peaking at about 680 nm is typical for the chlorophyll *a*-related oxidation products of bchl *b* [18]. Similar, albeit irreversible spectral changes have now been observed upon (partial) proteolysis of *E. halochloris* membranes. Here we wish to report results pertaining to the orientation and exposition of the photosynthetic membrane proteins of this organism, and combine data from SDS-gel electrophoresis and spectroscopy,

Abbreviations: Rp, *Rhodopseudomonas;* Rs, *Rhodospirillum;* E, *Ectothiorhodospira;* bchl, bacteriochlorophyll; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonylfluoride; EDTA, ethylendiamintetraacetate; cd, circular dichroism; LHP, light-harvesting-protein; RC, reaction center.

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in order to relate spectrally distinct chromophores to certain polypeptides.

Material and Methods

E. halochloris was grown anaerobically in the medium of Imhoff and Trüper, with the differences described earlier [17]. The cells were harvested by centrifugation (14000 × *g*) and washed once with Tris-buffer (10 mM, pH = 7.5). Thylakoids in which mainly the cytoplasmic surface is exposed, were prepared by the method of Feher and Okamura [19] and checked by light microscopy for homogeneity. Spheroplasts (rightside-out particles) were prepared after a modified method of Michels and Konings [20]: Harvested and washed cells were homogenized in a glass potter in Tris-buffer. The crude extract was treated with lysozyme (0.5 mg/ml) and EDTA (final concentration 5.5 mM) and stirred at room temperature for about 3 hours [21]. It is necessary to check the formation of the particles by microscopy (characteristic swelling; addition of more lysozyme if necessary). The suspension was then sonicated twice for one minute, centrifuged twice like chromatophores and adjusted to an absorption of 50 (1020 nm; 1 cm cuvettes).

For controlled proteolytic digestion, 500 µl of the membrane particles (chromatophores or spheroplasts) were incubated with increasing amounts of different proteases: proteinase K, trypsin or thermolysin. The digestion was stopped with PMSF (2 ml saturated solution in acetone/0.5 ml incubation mixture), trypsin-inhibitor (400 mg/ml incubation buffer, added as a solid) or EDTA (1.0 M final concentration), respectively. The samples were then centrifuged (14000 × *g*) washed three times with Tris-buffer and analyzed.

SDS-gel electrophoresis was done on polyacrylamide gels (PAGE) with a linear gradient (11.5–16.5% acrylamide), modified from Laemmli [22], as described earlier [17]. For calibration a standard set of hydrophilic proteins was used (bovine serum albumin; hen egg albumin; lactoglobulin; pepsin; trypsinogen and lysozyme) and in addition hydrophobic peptides (*Rp. spheroides* B 800/850 antenna) of known molecular weights [23]. Gels were scanned after staining with Coomassie brilliant blue G on a scanner TCD (Vitatron).

Absorption spectra were measured on a DMR 22 (Zeiss, Oberkochen) or a ZWS II spectrophotometer (Sigma, Berlin) connected to a BS 8000 intelligent recorder (Bryans, Mitcham). CD-spectra were obtained on a dichograph V (ISA, Unterhaching) equipped with a silex data handling system (Leanord, Lille) with a modified software. Fluorescence-emission spectra were obtained on a home-built fluorimeter equipped with a liquid-helium cryostat as described elsewhere [24] and are uncorrected.

All chemicals were reagent grade. Trypsin and proteinase K were purchased from Merck, Darmstadt, lysozyme and trypsin inhibitor from Serva, Heidelberg, thermolysin from Boehringer, Mannheim, and the SDS-PAGE calibration set from Sigma, München.

Results

Absorption spectra

E. halochloris has two major near infrared absorptions at 1020 and 800 nm. When thylakoids are treated with proteases, the 1020 nm absorption is gradually transformed into a 960 nm absorption, whereas the 800/830 nm band remains unchanged (Fig. 1). Qualitatively these changes are identical irrespective of the type of protease used (trypsin, proteinase K or

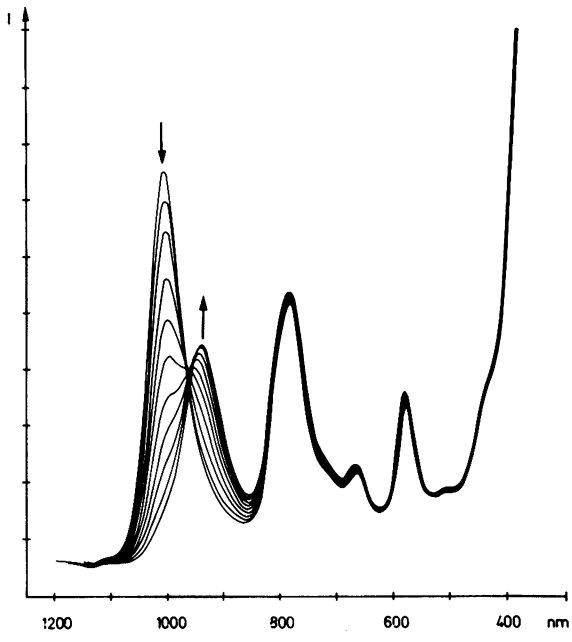


Fig. 1. Titration of *E. halochloris* thylakoids with increasing amounts of trypsin in 10 mM Tris buffer, pH = 8.0. The final concentration of trypsin was 100 µg/2 ml thylakoid suspension with A1020 = 0.7/cm.

thermolysin). The absorption changes are similar to the ones observed earlier upon lowering the pH [17], with two differences: Firstly the reaction is irreversible and thus due to a true proteolysis and not a pH-change induced by any action of the enzymes. Secondly the effectiveness of proteolysis is strongly dependent on the membrane orientation. In contrast to the acid induced absorption change, proteolysis works with thylakoids only, but not with spheroplasts. Similar to the pH-changes, the 960 nm form is again only metastable and transforms within a few minutes to yield the chlorophyll *a*-related oxidation-products of bchl *b* (Fig. 2) [18].

There is yet a third means of inducing the 1020 → 960 nm transformation in *E. halochloris*, *e.g.* by the treatment with urea. Globular, hydrophilic proteins are generally fully denatured (unfolded) by treatment with 8 M urea. The membrane proteins of *E. halochloris* are much more stable, if judged from their absorption spectra ≤ 900 nm. However, the 1020 nm band is again sensitive and transformed increasingly rapidly with increasing urea concentrations (4–8 M) to the 960 nm form. The absorption changes (Fig. 3) are identical to the ones induced by proteolysis or acid. Only thylakoids are susceptible to this treatment which is similar to the results obtained upon proteolysis. We have also been unable to reverse the urea-induced transformation. Possibly the time necessary to remove the denaturant (*e.g.* 10 min necessary for by filtration over a short column of desalting gel) is already too long to prevent the further and principally irreversible oxidation of the bchl *b* absorbing at 960 nm.

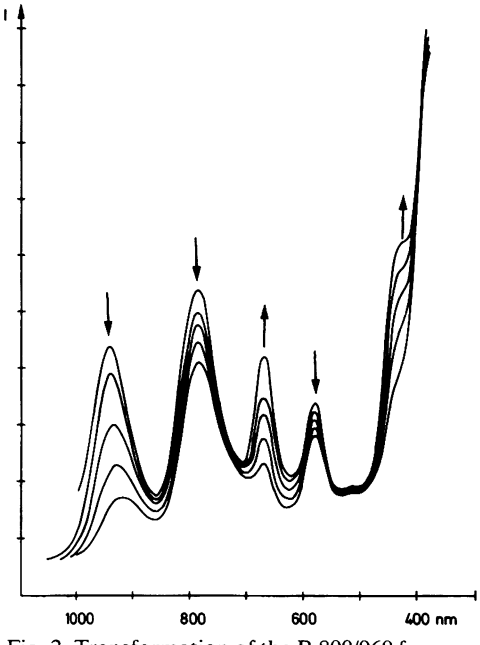


Fig. 2. Transformation of the B 800/960 form upon standing to the "B" 800/680 form. Sample as in Fig. 1. The time between each curve was 5 minutes.

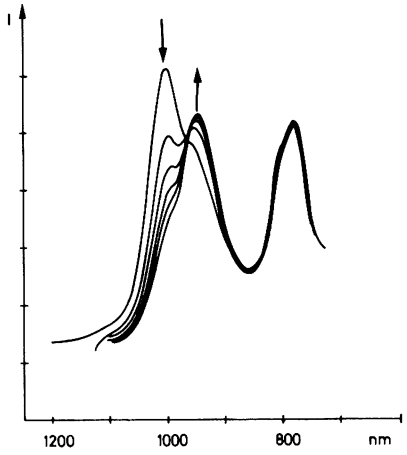


Fig. 3. Titration of *E. halochloris* thylakoids with increasing concentrations of urea (5.0, 5.5, ..., 7.5 M urea) in 10 mM Tris buffer, pH = 8.0.

Fluorescence spectra

In order to further compare the 960 nm forms obtained by the different treatments (acid-, urea- or protease), low-temperature fluorescence spectra were recorded:

The low temperature (5 K) emission spectra of all three forms show a single band peaking at 1007 nm (5 K), whereas the original emission of *E. halochloris* is at 1066 nm (Fig. 4). If the bacteriochlorophyll *b* chromophores absorbing at longest wavelengths are the emitters, this corresponds to Stokes-shifts of about 46 nm, for both the original chromophores (λ_{max}, absorption = 1020 nm) and the modified ones (λ_{max}, absorption = 960 nm). The fluorescence excitation spectra below 920 nm are identical for all four samples with respect to band positions and intensities (Fig. 5). This is further support that the chromophores absorbing at 800/830 nm are not affected by any of these treatments. In all three forms, there is also an efficient energy transfer from the 800/830 nm absorbing chromophores to the 960 nm ones, if judged from the excitation peak around 800 nm for

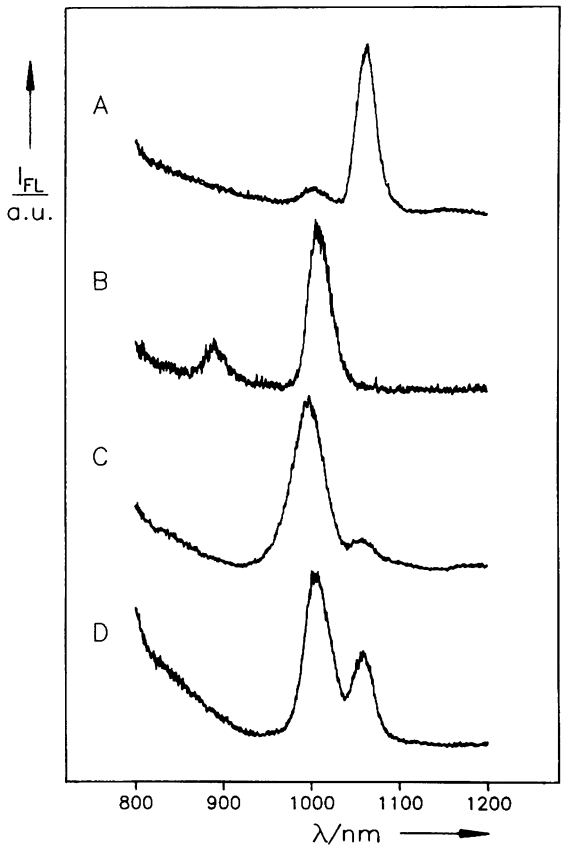


Fig. 4. Fluorescence emission spectra (excitation: 600 nm) of *E. halochloris* chromatophores at 5 K. A. Untreated chromatophores (λ_{max}, absorption = 1020 nm); B. acid induced "low-pH" form (λ_{max}, absorption = 960 nm); C. protease treated (λ_{max}, absorption = 960 nm); D. urea treated (λ_{max}, absorption = 960 nm).

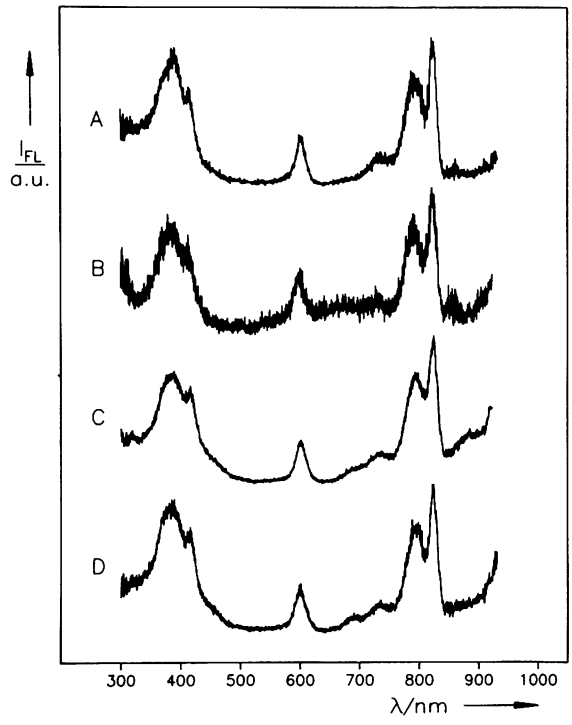


Fig. 5. Excitation spectra, corresponding to the emission spectra in Fig. 4. Emission at 1020 (A) and 960 nm (B, C, D), respectively.

the 1007 nm emission. In the visible and near-UV spectral range, the excitation spectra are indicative of (monomeric) bacteriochlorophyll *b* and the absence of bacteriopheophytin *b* (no bands around 530 nm).

Circular dichroism

The three species modified by either low pH, proteolysis or urea are also identical with respect to their circular dichroism spectra. All three 960 nm forms show an "S"-shaped band centered around 974 nm with extrema around 990 and 940 nm (data not shown). As discussed previously for the low-pH form [17], these spectra can be rationalized by (a minimum of) two strongly interacting bchl *b* molecules absorbing around 960 nm.

The 800/830 nm region shows an M-shaped signal, which is related to (a minimum of) three chromophores. The magnitude of this band is the only indication, that at least some changes take place, too, in the pigments absorbing around 800 nm during the transformation of the B 800/1020 to the B 800/960 forms. The cd pattern of the latter in the 800 nm region is similar in shape, but reduced in intensity by about 50% as compared to the 800/1020 nm form.

SDS-gel electrophoresis

In order to relate distinct changes in the polypeptide pattern to the spectroscopic changes and hence to chromophores with distinct absorptions, the incubation with protease was followed in parallel by SDS-polyacrylamide gel electrophoresis and absorption spectroscopy. The SDS-PAGE of *E. halochloris* membranes shows four bands in the "high-molecular weight" range ≥ 16 kDa (34.0, 28.0, 23.8 and 16.8 kDa). They have similar relative, but generally higher mobilities than the polypeptides of the reaction center polypeptides from *Rp. viridis* (Table I)

and were therefore tentatively assigned to the RC subunits cytochrome *c*, H, M and L.

Table.

Rp. viridis (a)		*E. halochloris* (c)
38.0	cytochrome *c*	34.0
33.0	H	28.0
27.0	M	23.8
24.0	L	16.8
	?	15.2
	?	14.5
11.0 (6.848, b)	α-LHP	13.5 (6.5, d)
8.0 (6.138, b)	β-LHP	13.0 (6.0, d)
6.0 (4.001, b)	γ-LHP	12.2 (4.5, d)

(a) SDS-PAGE data from Jay *et al.*, 1983 [25].
(b) Data from primary structure analysis [26].
(c) Data from SDS-PAGE (this work), calibrated with hydrophilic globular proteins.
(d) Calibrated with B 800/850 subunits from *Rp. sphaeroides*.

Fig. 7. SDS-PAGE of trypsin incubate chromatophores of *E. halochloris*, that shows the degregation of the 6.5 kDa polypeptide. 0.5 ml chromatophores (E 1020 = 50) were incubated with 650, 700, 750, 800, 900, 1000 µg trypsin from left to right for 30 minutes at room temperature. The assignments of the main bands is indicated on the left margin. See text for the molecular weights.

In the low-molecular weight region (≤ 16 kDa) two barely resolved major bands appear in addition to a weakly staining third band (Figs. 6, 7). Since they are isolated with the antenna fraction, they have been assigned to the light-harvesting complex [17]. This composition is again similar to that of *Rp. viridis* (Table I). The only exception is that a 28.0 kDa band is isolated with the antenna from *E. halochloris*, whereas a polypeptide of this size is generally isolated as the "H"-subunit of the reaction center [17].

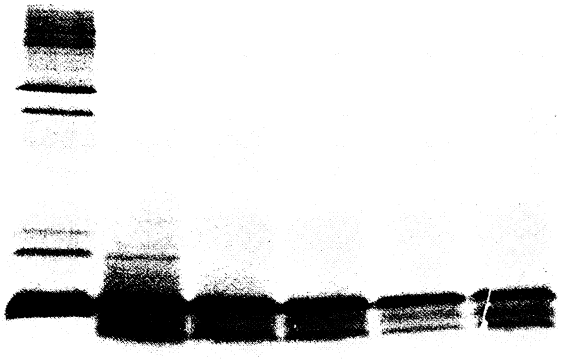


Fig. 6. SDS-PAGE of chromatophores from *E. halochloris*, incubated with proteinase K. 3 ml of the chromatophores (E 1020 = 50) were incubated with 0.5 mg proteinase K for 0, 5, 15, 30, 45 and 75 from left to right minutes at room temperature. See Fig. 7 for assignment of the bands.

The apparent molecular weights of these membrane proteins are shown in Table I.

When thylakoids of *E. halochloris* are incubated with increasing amounts of proteases, the polypeptide bands were degraded in a specific sequence. SDS-PAGE gels of the digestion with trypsin are shown in Fig. 6, but similar results are obtained with proteinase K and thermolysin (not shown).

In the "high molecular weight" region the cytochrome band disappears first, followed rapidly by H, L, and much more slowly by M. This means that in the membrane the M subunit is the most stable peptide of the RC (within the limits of resolution of our gels, *viz.* ± 5 amino acid residues). Since the sequence of digestion is the same with all three proteases used, in a first approximation only the accessibility of the proteins is important rather than a distinct amino-acid sequence. It should be noted that in the bchl *a* containing organisms *Rp. capsulata* [27] and *Rs. rubrum* [28, 29] the L subunit of the RC is the most stable one. However, the assignment of RC bands by mobility alone is insufficient and further confirmation of this assignment is necessary.

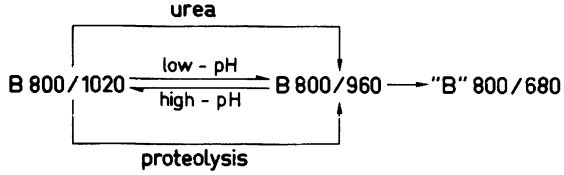
The lower-molecular-weight polypeptides of the light-harvesting complex were digested much slower than the RC polypeptides. This different time course of the digestion has helped us to assign the fragments

to either the RC or LH polypeptides. A gel with high resolution in this region is shown in Fig. 6. The 6.0 kDa band is least stable. It is degraded only a little to yield a band with an apparent molecular weight of 5.3 kDa, while the intensity of the 6.5 kDa band remains constant. Secondly, the 6.5 kDa band is attacked. The fate of the fastest migrating γ-peptide (4.5 kDa) is difficult to assess quantitatively, because it stains only weakly with Coomassie blue. When incubating the isolated antenna complex of *E. halochloris* all bands disappear more or less simultaneously. This can be rationalized by the protection of the hydrophobic surfaces of the peptides in the chromatophore membrane which is lost in the solubilized complex.

Discussion

The spectroscopic results suggest, that the different treatments of *E. halochloris* membranes, *e.g.* lowering of the pH below 6.5 [17], proteolysis and incubation with urea, transform the pigment arrangement within the antenna apparatus in the same or at least in a very similar manner. Of these reactions, only the one induced by acid is reversible. A reversion may principally be possible, too, for the treatment with urea. However, the fastest time achieved for its removal (≈ 10 min) is comparable to the half-life of the 960 nm chromophores and turned out to be ineffective. These results are summarized in Scheme 1.

The proteolysis has been studied in more detail. Two distinct results regarding the antenna structure within the membrane of *E. halochloris* can be drawn. The first is a correlation of the chromophores absorbing at different wavelengths to distinct polypeptides. The absorption shift from 1020 to 960 nm occurs simultaneous to the digestion of the 6.5 kDa (= α-) subunit, and after the proteolysis of the 6.0 kDa antenna polypeptide and any of the reaction center polypeptides. The chromophores absorbing at 1020 nm in the native antenna are thus either bound



Scheme 1.

to the α-subunit or their spatial arrangement is at least strongly influenced by it. The role of the γ-subunit is difficult to assess because it stains only weakly and unreliably. Like in *Rp. viridis* [26] it does not carry a histidine residue (Brunisholz, unpublished). Since histidine is currently assumed to be the common binding amino acid for the chromophore in the bacterial antenna polypeptides [23, 30, 31], the γ-subunit is considered a structural polypeptide, to which the crystallinity of the membranes [10–12] in bacteriochlorophyll *b*-containing bacteria may be related [26]. The chromophores absorbing around 800 nm should then be bound to the other antenna polypeptides, most likely to the β-subunit (6.0 kDa). However, proteolytic cleavage of a small (≈ 0.5 kDa) oligopeptide from this subunit does not affect significantly the 800/830 nm absorption.

The relative intensities of the chromophores absorbing at 1020 to the ones absorbing at 800 nm is roughly 2:1 and thus similar to the relative intensities of the chromophores absorbing at 850 and 800 nm, respectively, in type I B 800/850 antenna complexes of bchl *a*-containing species, *e.g. Rp. spheroides* [32]. In both complexes, the chromophores absorbing at the longest wavelengths are bound to the heavy antenna polypeptide [23]. The most significant difference is the coupling of the chromophores inferred from the circular dichroism spectra. In the B 800/850 complex of *Rp. spheroides*, four excitation coupled bacteriochlorophyll *a* molecules were discussed as being responsible for the 850 nm absorption and orientated parallel to the α-helix of the polypeptides, and two weakly coupled chromophores orientated perpendicular for the 800 nm absorption [33]. In *E. halochloris*, a minimum of three strongly coupled chromophores is responsible for the 800 nm band and at least 2 for the 1020 nm absorption [17]. No data are available on their orientation with respect to the membrane.

The second aspect of the proteolytic digestion concerns the topology of the antenna of *E. halochloris*. Only the H-subunit of the reaction center is digested if spheroplasts are treated with proteases. In thylakoids having mainly the cytoplasmic side exposed, all polypeptides of the antenna (with the possible exception of the weakly staining γ-subunit) and all reaction center polypeptides are accessible to proteases. This would indicate, that only the H-subunit is spanning the photosynthetic membrane, as far as its accessibility to proteases is concerned. Similar

conclusions have been drawn from proteolytic studies with the bchl *a* containing *Rs. rubrum* [8, 9, 34, 35], *Rp. spheroides* [36] and *Rp. capsulata* [5, 27], as well as with the bchl *b*-containing *Rp. viridis* [34]. In all cases, the reaction center polypeptides are more labile than the ones related to the antennas, and only the H-subunit has always been found to be accessible from either side of the membrane. The validity of the latter results has been questioned, however, by the x-ray data of Deisenhofer *et al.* [13] on *Rp. viridis* reaction centers. The major part of the H-subunit is located on the cytoplasmic surface, with only a single α-helix spanning the membrane and less than 10 amino acid residues being exposed on the periplasmic side. From the x-ray data, it is rather the L- and M-subunits which are transmembrane polypeptides. These discrepancies may be due to (i) a true species difference, (ii) a misassignment of the H band from SDS-PAGE derived molecular weights, or (iii) a fortuitous overlap of a large H fragment with either the M or L band. The third possibility should be indicated by an increased intensity of either the M or L band, which was not observed. The second possibility can presently not be decided upon for the lack of sufficient sequence data. Species differences are indicated by differential proteolytic sensitivities of polypeptides of the photosynthetic membranes of the species cited above. They are also supported by labeling methods exhibiting different selectivities or steric requirements, *e.g.* by iodination [2, 5, 25, 35] and by immunochemical data [5, 25, 39]. However, a comparison of the results obtained in different laboratories and with different biochemical techniques is difficult, and comparative work with different species under otherwise identical conditions is necessary. Such work with *E. halochloris* and the much better known *Rp. viridis* is in progress.

Acknowledgements

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